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Award Number: DAMD17-02-1-0407

TITLE: Novel Role of ANX7 in Breast Cancer Progression

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REPORT DATE: June 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2003	Annual (9 May 02 - 8 May 03)

4. TITLE AND SUBTITLE

Novel Role of ANX7 in Breast Cancer Progression

5. FUNDING NUMBERS

DAMD17-02-1-0407

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The *ANX7* gene codes for a Ca^{2+}-activated GTPase, and is a tumor suppressor gene *ANX7* expression has prognostic value for predicting survival of breast cancer patients. Our **objective** in this proposal is to determine the mechanism and the signaling pathway by which the *ANX7* gene induces death of breast cancer cells. To this end, we have generated and identified the dominant negative mutants against calcium and GTP in the anx7 coding domain which are necessary for biochemical functions of ANX7. We also have generated the effective adenoviral constructs containing the dominant negative mutants and wt-ANX7 and have shown that altered ANX7 expression during breast cancer cell growth involves calcium. Additionally, we confirmed the role of calcium by determining ANX7's control on all three subtypes of IP3 Receptor expression. Using cDNA microarray and Western blot analysis, we have identified the downstream targets and signaling pathway of ANX7 in apoptosis and suppression of breast cancer cell growth. Taken together, these data indicate that ANX7 suppresses breast cancer cell growth and calcium plays a role via IP3-Receptor. In addition, we have identified the members of the ANX7 signaling pathway. Comprehensive effort is underway to validate the results that will further elucidate the ANX7 dependent calcium signaling pathway in breast cancer cells.

14. SUBJECT TERMS

ANX7, calcium, IP3 receptor, signal transduction, cDNA microarray

15. NUMBER OF PAGES

18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Current attempts to develop more effective therapies for breast cancer have been focussed on the discovery of new tumor suppressor genes, in hopes of using them for gene therapy. We have recently found that the *ANX7* gene, which we discovered and have continued to study in the context of Ca^{2+} and GTP mediated exocytosis for many years (Creutz et al, 1978; ibid, 1979; Raynal and Pollard, 1994; Caohuy et al, 1996; Srivastava et al, 1996), is defective in 35% of human prostate cancer specimens (Srivastava et al., 2001) and 40% of human breast cancer specimens. A detailed analysis of ANX7 levels in hundreds of breast cancer specimens reveals that expression of this candidate tumor suppressor gene is specifically altered in primary breast cancers, ductal carcinoma in situ (DCIS) and invasive breast cancers. Furthermore, high cytoplasmic expression of *ANX7*, is a predictor of reduced disease-free survival (Srivastava et al., 2001). These data therefore, strongly suggest that the *ANX7* gene has clinical relevance for breast cancer in women. This is an important insight because until now the *ANX7* gene has never been thought to play such a role (Raynal and Pollard, 1994). In our preliminary studies with metastatic estrogen receptor positive breast cancer cells in vitro, we find that *ANX7* gene therapy causes these cells to undergo apoptosis, or programmed cell death. Therefore our **objective** in this proposal is to determine the mechanism and the signaling pathway by which the *ANX7* gene induces death of breast cancer cells. The **rationale** behind this study is the hope that such knowledge will enable us to develop therapies based on the use of the *ANX7* gene to treat or prevent breast cancer.

Our studies with both human cancer cells and the Anx7 knockout mouse have indicated the existence of a possible common deficit in calcium regulation (Srivastava et al., 1999). In the case of cancer cells, it is known that the initial signal for the onset of programmed cell death is the release of a pulse of calcium from the internal calcium stores in the endoplasmic reticulum. The calcium is released into the cytoplasm through a protein channel in the endoplasmic reticulum membrane called the IP3-Receptor. A hint regarding the possible connection between the ANX7 gene and the calcium pulse, comes from our recent work on the *ANX7* (+/-) knockout mouse. Tissues in this mouse express low levels of Anx7 protein as well as low levels of IP3-Receptors in the endoplasmic reticulum. Intracellular calcium signaling is accordingly defective, in spite of normal levels of extracellular calcium. We are reminded that apoptosis, from whatever cause, is known to be suppressed in the absence of IP3-Receptors. We also found that the effect of the anx7 gene on the DU145 prostate cancer cell line enhanced the capacity for undergoing apoptosis in the presence of staurosporine. **On the basis of these results we have hypothesized that the ANX7 gene kills breast cancer cells by increasing IP3-Receptor expression, thereby potentiating the IP3-dependent apoptotic calcium signaling pathway.**

BODY

Reason for the change in the statement of work for Aim #1 and 2. To conduct experiments efficiently in a timely manner, first we concentrated in accomplishing the SOW 2.a. That is to test sites of Ca^{2+}, GTP and PKC on ANX7 for importance in induction of apoptosis. Since we can test both the wild type and best characterized mutants against calcium, GTP and PKC for apoptosis and tumor suppression simultaneously and accomplish SOW 1.a, I request a change in the time line for Aim #1 and 2

STATEMENT OF THE THE WORK:

TIME-LINE FOR EXPERIMENTAL PLAN (OLD PLAN)

	YEAR #1	YEAR #2	YEAR#3
Aim#1 and 2.			
1.a. Test tumor cells for biochemical characteristics of apoptosis	----------		
2.a. Test sites of Ca^{2+}, GTP and PKC on ANX7 for importance in induction of apoptosis		----------	
2.b. Test tumorigenicity of MCF7 cells expressing wildtype and mutant ANX7 in cancer cells			----------

TIME-LINE FOR EXPERIMENTAL PLAN (NEW PLAN)

	YEAR #1	YEAR #2	YEAR#3
Aim#1 and 2.			
1.a. Test sites of Ca^{2+}, GTP and PKC on ANX7 for importance in induction of apoptosis	----------		
2.a. Test tumor cells for biochemical characteristics of apoptosis		----------	
2.b. Test tumorigenicity of MCF7 cells expressing wildtype and mutant ANX7 in cancer cells			----------

Milestone #1: Elucidation of the relationship between *ANX7*-induced alteration of apoptosis and tumor suppressor activity in breast cancer cells
Milestone #2: Defining the biochemical requirements and regions in ANX7 that confer apoptosis and tumor suppressor activity in breast cancer cells

In the first year of the grant proposal we have focused on the generation of adenovirus constructs containing wild type ANX7 and the dominant negative mutant against calcium and GTP. We investigated the anticarcinogenic effects of the altered ANX7 expression **(SOW for Aim #1 and 2)** and studied the expression of all three IP3-Recetors **(SOW for Aim #3)**. We also began studies on the signaling pathway using cDNA microarray to gain possible mechanism for ANX7's inhibition of growth **(SOW for Aim 4)**..

Statement of Work. 1.a.

To define the biochemical requirements of ANX7 for apoptosis and tumor suppressor activity in breast cancer cells, we will determine the "hot spot(s)" in the anx7 coding domain which are necessary for tumor cell proliferation and apoptosis.

The transformants with the 'tet off' system (from Clonetech) bearing wildtype ANX7 gene had problem of leakage in the absence of tetracycline. So we switched to adenoviral system. *ANX7* is expressed at very low levels in breast cancer cells. Hence it is critical to generate adenoviral expression vectors which has high transfection efficiency to study the biological functions of *ANX7*. To determine the involvement of calcium, GTP and PKC, we first generated mutations at those sites in mammalian pTrc99 vector and tested in vitro the ANX7 activity for membrane fusion. Therefore, we generated expression vectors containing wt-ANX7, 16 mutants against calcium binding site, 8 mutants against GTP binding site and 3 mutants against PKC binding site to study the biological functions of ANX7. The selected dominant negative mutants were reconstructed into adenovirus system and used for its activity for tumor suppression, apoptosis and IP3-Receptor expression and other downstream targets and were compared with wt-ANX7.

1.a.1. Generation of dominant negative mutants of ANX7.

Rationale: Dominant negative mutants of tumor suppressor genes have been useful for investigating the mechanism of action of these genes. Since it is well known that ANX7 forms Ca^{2+}-dependent polymers as a prelude to membrane interactions, it seemed reasonable to anticipate that mutations affecting Ca^{2+} binding might have the ability to generate partially crippled ANX7 monomers in the polymerization reaction. We reasoned that if a mutant could be found that inhibited polymerization, we could test whether tumor suppressor activity depended on this process.

Hypothesis: Mutations at some or all of the four Ca^{2+} binding sites on ANX7 may act as dominant negative mutants.

Experiment: Using standard techniques, site directed mutations were introduced into the calcium binding sites in combinations of all four crystallographically defined endonexin fold motifs. All four have the consensus sequence [GXGTDE] and the mutations were engineered to generate the amidated analogues of the charged residues (*viz.*, [GXGTNQ]). Thus we prepared 16 different combinations, including the wildtype ANX7. The combinations were single mutations (e.g., 1, 2, 3 or 4); mutations at two sites (e.g., 1 & 2, 1 & 3, etc); mutations at three sites (e.g., 1&2&3, 2&3&4,etc) and all four sites (e.g., 1&2&3&4). All the mutants were prepared and tested in the phosphatidylserine liposome fusion assay. Some were as active as the wildtype, while

others were much less active. As shown in **Figure 1**, one mutant, ANX7J, was both intrinsically inactive, and profoundly inhibitory when mixed with equi-molar amounts of wildtype ANX7 (*viz.*, 1 μg each of ANX7 proteins).

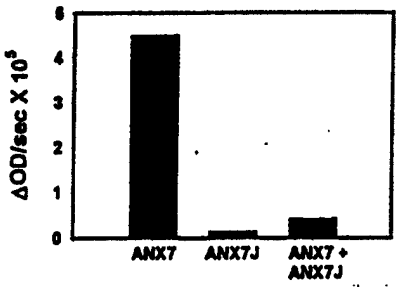


Figure 1: Dominant negative activity of **ANX7J** mutant when mixed with wild type ANX7 in *in vitro* membrane fusion assay.

Interpretation: ANX7J behaves as a dominant negative mutant in the *in vitro* test, and is now ready for testing for tumor suppressor activity. Inasmuch as the anx7(-/-) mouse mutant is embryonically lethal, we are prepared for the possibility that this mutation may prove to be cytotoxic.

1.a.2. Generation of GTP-binding site mutations in human anx7

Rationale: ANX7 is a Ca^{2+}-activated GTPase, which contains the five putative RAS-type canonical GTP binding sites. We reasoned that mutating these sites might allow us to test whether GTP might direct any activities of ANX7, including tumor suppression activity.

Approach: Since we did not know which mutations in these GTPase domains might be important for ANX7 activity, we created and expressed mutant ANX7's containing discrete site-directed RAS-like mutations. These sites in ANX7 were G-2 (QinT); G-4 (NRsN); and G-5 (EiSG). Binding of 8-azido-GTP could then be used to assess GTP binding.

Experiment: Mutations were generated in the putative G-2 Effector or GAP domain either as T148*A (termed TA1, mutation in the cassette exon#6), or as a double mutation,[T148*A, T148A] (termed TA2, with an additional mutation in Exon #7). Other mutations included: a G-4 mutation, N192I (termed NI in **Figure 2**); another G-4 mutation, N195D (termed ND in **Figure 2**); a G-5 mutation, L221I (termed LI in **Figure 2**). One final mutation, F233L (termed FL in **Figure 2**), a conserved site in all *ras* superfamily members, was also prepared. In *ras* this mutation disorders the GTP binding pocket.

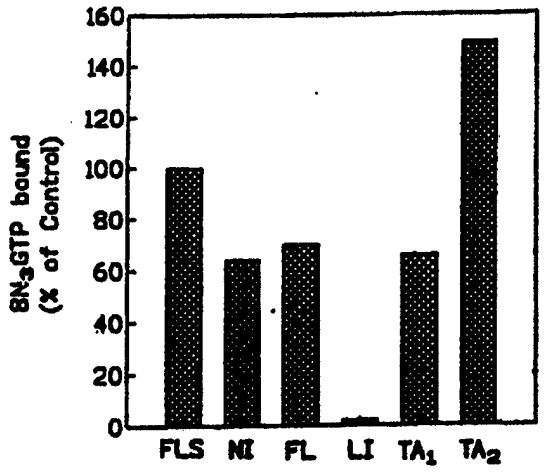


Figure 2: 8-azidoGTP photoaffinity labeling of ANX7 mutated at putative Ras-like consensus GTP-binding sites.

As shown in **Figure 2**, Western blots showed that substantial amounts of mutant proteins could be prepared. The PhosphoImager data, as well as the graphical

representation below, reveal that the LI mutation entirely blocks GTP binding, while NI, TA1 and FL mutants are approximately 60% active. By contrast, the TA2 mutation is approximately 50% activated. "FLS" represents recombinant full-length synexin, or ANX7. Recall that the TA1 and TA2 mutations are in a higher molecular weight ANX7 isoform containing the cassette exon #6, and for that reason run slower on the SDS gel. In RAS, the equivalent LI mutation prevents GTP from binding, just as it does in ANX7.

Interpretation: These data serve to validate the structural basis of the intrinsic GTPase activity of ANX7, and provide mutant anx7 sequences that can be used to assess the importance of the GTPase or GTP binding for tumor suppressor gene activity.

Methods: The wildtype and mutant ANX7 proteins were expressed in the pTrc99A expression system in *E.coli*, and purified to ca. 90% by differential ammonium sulfate precipitation and column chromatography on Ultragel AcA54 (see Cauhuy et al, 1996 for more details). Specific ANX7 content of the 47KDa or the 51KDa bands were estimated by using the ^{125}[I] anti-mouse IgG secondary antibody to label transblotted samples on nitrocellulose that had been bound by primary monoclonal antibody 10E7. ANX7 and ANX7 mutants were photolabeled by 8-N_3-^{32}[P]-GTP in the presence of 2 mM glutathione to block non-specific binding presently available in our laboratory.

1.a.3. Generation of PKC-binding site mutations in human anx7

Rationale: ANX7 is a Ca^{2+}-activated GTPase, which is activated by PKC and contains two PKC binding sites. We reasoned that mutating these sites might allow us to test whether PKC might direct any activities of ANX7, including tumor suppression activity.

Experiment: Since we did not know which mutations in these PKC binding domains might be important for ANX7 activity, we created and expressed single mutants of ANX7 in each PKC binding site and created double mutants at those sites. We measured the phosphorylation of ANX7 by PKC and found that the double mutants worked the best.

Statement of Work. 2.a.

2.a. Generation of adenoviral vectors containing wild type *ANX7* and mutants of *ANX7* in calcium, PKC and GTP binding sites and its action on cell proliferation.

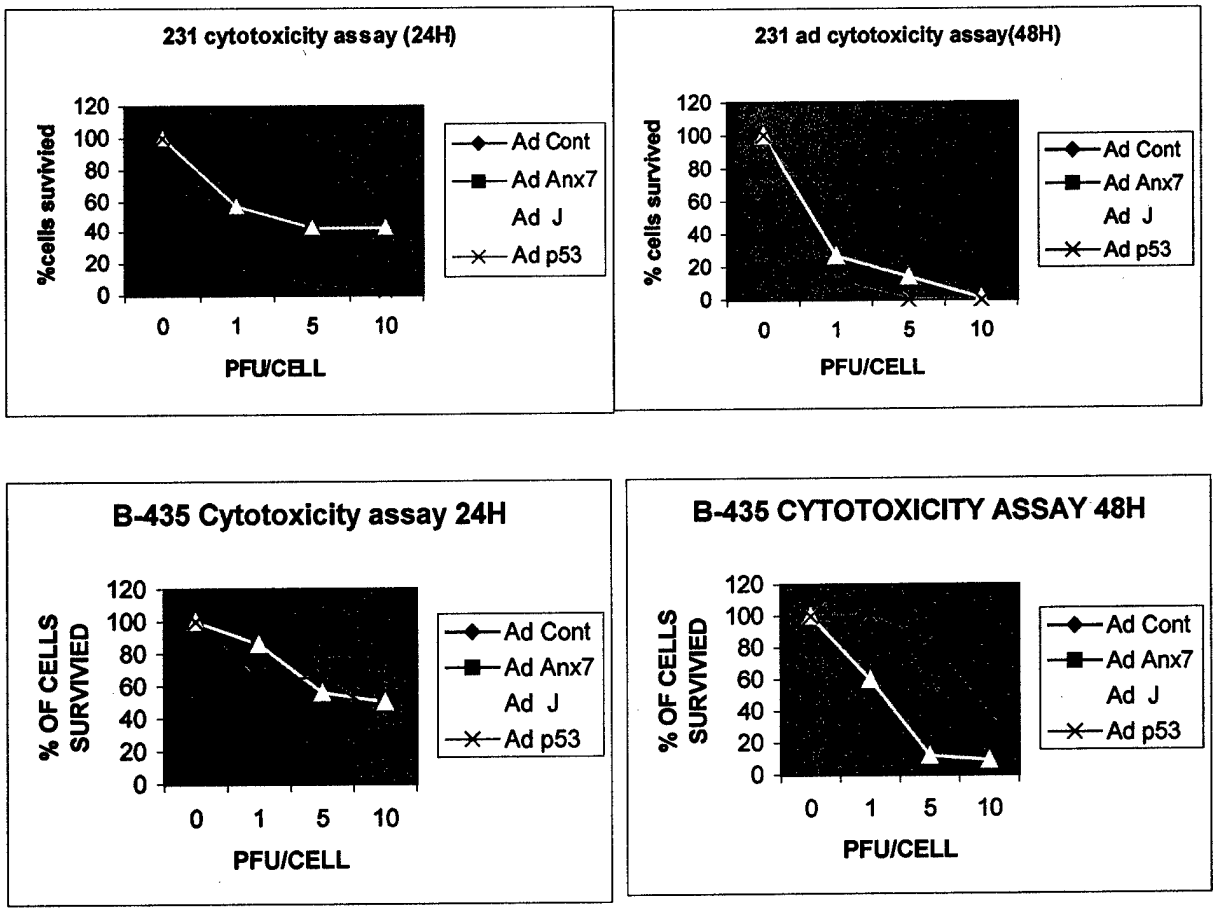
Rationale: The underlying hypothesis upon which this aim is based is that the tumor suppression property can be traced to specific "hot spot" amino acid residues in the ANX7 protein. We also hypothesize that those residues that control tumor cell growth in vitro will also control tumor cell proliferation *in vivo*. To test this hypothesis we propose to begin by testing the effect of identified mutations in sites which known to affect anx7 functions such as Ca^{2+} binding, PKC binding, GTP binding and hydrolysis. For both in vitro and in vivo cases we also propose to test the effect of constructs which lead to complete loss of function, including antisense anx7 and a dominant negative anx7 mutation. If anx7 is a tumor suppressor gene, then specific residues in the ANX7 protein ought to exist which are important for the function of suppressing tumor cell proliferation and apoptosis

Approach: We have prepared ANX7 genes in a recombinant adenovirus expression system which contain tested mutations affecting different ANX7 functions. These sites

include Ca^{2+} binding and GTP binding and hydrolysis. It is quite logical to anticipate that any or all of these functions might be important for suppressing the growth of tumor cells. These mutations are described in section **SOW. 1.a.** We took the dominant negative mutant ANX7J and carried out cytotoxicity assay in breast cancer cells

Experiment: Adenovirus vectors containing either the wildtype anx7 gene or the dominant negative mutant ANX7J were transfected into the non-metastatic (MDA-MB-231) and metastatic (MDA-MB-435) breast cancer cell lines and tested for growth inhibition. Initially, the cells were infected with 1, 5 or 10pfu/cell of either control adenovirus, or adenovirus expressing wildtype or mutant anx7. The cells were analyzed for their growth at different times after infection. Uninfected cells were analyzed in parallel. Cell growth was monitored and counted using a hemocytometer at 24 and 48 hours. The results which were obtained with trypan blue experiment previously reported had been carried out with adenovirus which was freeze thawed several times and was not giving reproducible results. So, we prepared and purified new batches of adenovectors containing wt-ANX7, ANX7J and p53 at the same time and stored several aliquots in the freezer so there is no discrepancy in the results. We used p53 as a positive control. The experiments were carried out in triplicate. The cytotoxicity assay revealed that in non-metastatic 231 cells, at 24 hours wt-ANX7 and the dominant negative mutant ANX7J had similar effect, while p53 addition had begun killing the cells. However, introduction of the dominant negative mutant ANX7J or p53 killed the cells very effectively even at 1pfu at 48 hours (**Figure 3**). These results suggest that the mutation at the calcium binding site has a role to play in killing the cancer cells.

Figure 3:



On the other hand, in the metastatic B-435 cells, at 24 hours p53 and the dominant negative mutant ANX7J behaved similarly, at 48 hours, the dominant negative mutant ANX7J killed the cancer cells effectively, while p53 did not. These results are in accordance with our finding with human clinical breast cancer specimens, since high expression of ANX7 was associated with the metastatic disease.

Interpretation: Since the dominant negative mutant ANX7J is against the calcium binding site and the addition of this mutant killed the cancer cells, we conclude that calcium associated function with this identified mutation is mechanistically involved in the tumor suppression phenotype showing metastasis. The effect of the dominant negative mutant ANX7J in killing the cancer cells implies that down-regulation of ANX7 activity could be therapeutic for breast cancer patients

Statement of Work. 3.a.

3.a. We show that the dominant negative ANX7 down regulated the IP3-Receptor expression in breast cancer cells

Rationale: The loss of up to 50-60% of endogenous ANX7 protein in the ANX7(+/-) knockout mouse results in a 10-fold loss of IP3 Receptors and complete loss of SOC channels. Since both IP3 Receptors and SOC channels are needed for activating apoptosis, and IP3 Receptor function is the physiological trigger for the mitochondrial permeability transition, it seems reasonable to expect that inactivating ANX7 in breast cells would down regulate IP3Receptors.

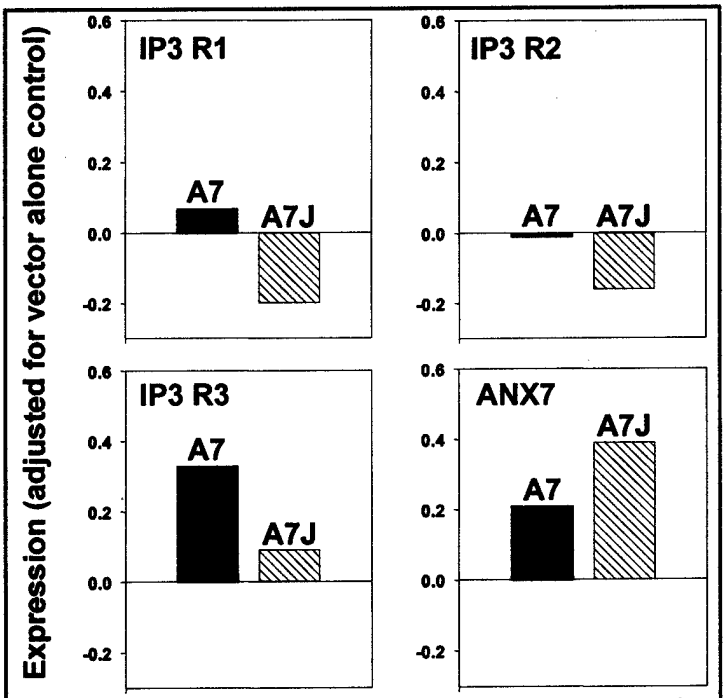


Figure 4: Influence of wild type ANX7 and dominanat negative ANX7J on IP3 Receptor subtype expression

Experiment: We measured ANX7 and IP3-Receptor mRNA levels in tumor cells treated with adenovirus vector alone, wildtype and dominant negative mutant ANX7. Quantitative RT-PCR was used to quantitate the IP3Receptor messages (types 1, 2 and 3), and levels of beta-actin message was used to normalize

levels of RNA used for the analyses. As is shown in **Figure 4**, dominant negative ANX7 down regulated all three IP3-Receptor expression in MCF-7 breast cancer cell line.

RT-PCR for IP3 Receptor mRNA subtypes: IP3-Receptors come in three subtypes, in which only a discrete domain varies among the three. The following HUMAN primers were prepared and tested in these experiments.

Type1= [FP:5'-CACCGCGGCAGAGATTGACAC-3'; RP: 5'-CCAGCTGCCCGGAGATTTC-3'

Type2= [FP: 5'-CTGGGGCCAACGCTAATACT-3'; RP: 5'-GAACCCCGTGATTACCTGTGACTG-3'];

Type3= [FP: 5'-GCGGGCCTGTGACACTCTACT-3'; RP: 5'-CGCCGCTCACCAGGGACAT-3'.

We found that Wt-ANX7 expression increased Ad-vector alone control from 0.49 to 0.70 and we observed corresponding increase in IP3R expression. While the dominant negative mutant ANX7J expression reached to 0.80, since it inhibited the activity of endogenous ANX7, we see a decrease in IP3R expression (**Figure 4**).

Interpretation: These results suggests that ANX7 controls all three subtypes of IP3 Receptor expression

Statement of Work. 4.a.

4.a.1. To identify the downstream targets of ANX7 that constitute the ANX7 signaling pathway by which ANX7 causes apoptosis and suppresses breast cancer cell growth.

Rationale: To investigate the underlying genomic mechanisms of wt-Anx7 action in breast cancer cells, we examined gene expression in breast cancer cells using cDNA microarrays obtained from clonetech. Our hypothesis is that we can assess **the downstream targets and signaling pathway** of ANX7 in apoptosis and suppression of breast cancer cell growth.

Experiment : In order to assess the downstream targets of ANX7 in breast cancer cells, we isolated mRNA's from parental breast cancer cells, breast cancer cell lines transfected with either

Table 1: List of genes that are most affected by *ANX7* and *p53* and least affected by *ANX7J*

Gene	parental	Vector alone	ANX7	Anx7J	P53
S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	0.916	0.961	0.586	0.967	0.564
splicing factor, arginine/serine-rich 7, 35kDa	0.031	0.230	-0.059	0.200	-0.128
c-mer proto-oncogene tyrosine kinase	0.068	-0.296	-0.034	-0.290	-0.253
KIAA0303 protein	0.031	-0.296	-0.034	-0.253	-0.061
immunoglobulin heavy constant gamma 3 (G3m marker)	1.111	2.638	0.881	1.864	2.098
proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)	-0.108	0.060	-0.143	0.100	-0.165
transforming growth factor, alpha	-0.166	-0.296	0.073	-0.218	-0.061
TRAF interacting protein	-0.108	0.167	-0.143	0.115	-0.003
cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	0.521	0.861	0.364	0.622	0.384
basigin (OK blood group)	1.238	1.680	1.111	1.458	1.296
gonadotropin-releasing hormone receptor	0.165	0.447	0.033	0.392	0.337
interleukin 14	-0.081	-0.338	-0.113	-0.429	-0.003
phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	0.010	0.602	-0.010	0.358	0.298
phosphoribosyl pyrophosphate synthetase 1	0.232	0.206	-0.010	0.259	0.024
pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1)	-0.011	0.206	-0.113	0.200	0.191
chromogranin B (secretogranin 1)	0.232	0.565	0.109	0.340	0.208
MCM2 minichromosome maintenance deficient 2, mitotin (S. cerevisiae)	0.010	0.352	0.053	0.270	0.155
nth endonuclease III-like 1 (E. coli)	1.002	1.167	0.872	1.416	0.613
growth arrest-specific 2	-0.233	-0.224	0.142	-0.332	-0.031
ribosomal protein L13a	0.936	1.007	0.797	1.265	0.312
pleckstrin homology, Sec7 and coiled/coil domains 2-like	0.332	0.745	0.427	0.646	0.578
aryl hydrocarbon receptor	0.031	-0.259	-0.010	-0.186	-0.128
tenascin R (restrictin, janusin)	0.459	-0.083	0.354	-0.031	-0.031
ribosomal protein S6 kinase, 90kDa, polypeptide 1	0.521	0.488	0.383	0.571	0.240
B-cell growth factor 1, 12kDa	0.165	0.361	-0.010	0.312	0.325

vector alone, wild-type, or dominant negative mutant ANX7J. We used ATLAS™ cDNA expression cancer array to obtain the expression profiles. Comparison of the transcripts between control, ANX7J and ANX7 wild type transfected tumor cells. Table 1 shows the results of the microarray data analysis described in Methods section below, revealed the genes that are most affected by wt-*ANX7* and *p53* and least affected by *ANX7J.* On top of the lists are genes related to calcium binding (S100 calcium binding protein), apoptosis and tumor suppression (TGF-beta) and cell cycle (p19, an inhibitor of CDK4).

Interpretation: The genes associated with the tumor varied with a diverse biological/biochemical functions linked to cell proliferation, apoptosis and tumorigenesis.

Methods:

Preparation and labeling of RNA

Total RNAs from breast cancer cells, either parental or transfected with either vector alone or ANX7, or p53 as a positive control were prepared by the method of Champenski et al. and were subjected to DNAse 1 digestion to eliminate genomic DNA contamination. Two rounds of purification of poly A+ RNA from total RNA were performed using the mRNA isolation kit from Invitrogen as recommended by the manufacturer. The quality of the RNA were tested by running a formaldehyde denatured agarose gel and quantitated by measuring the optical density at 260nm. A ^{32}P labeled cDNA probe was synthesized from 1 μg of poly A+ RNA from control and tumor samples using MMLV reverse transcriptase, dNTP mix and CDS primer mix comprising the oligonucleotide sequences for the 1200 cancer related genes spotted on the atlas cDNA microarray. The reaction was carried out in a thermocycler set at 50°C for 25 min. and terminated by the addition of 0.1M EDTA, pH 8.0 and 1mg/ml glycogen. The ^{32}P labeled cDNA probe was then purified from unincorporated ^{32}P labeled nucleotides by using a CHROMA SPIN-200 column (clonetech) as recommended by the manufacturer. The human atlas cDNA expression array containing 1200 cancer related genes on a nylon membrane was prehybridized using Express Hyb (clonetech) at 68°C for 1 hr and hybridized overnight at 68°C with the denatured and neutralized ^{32}P labeled cDNA probe. The membrane was washed three times with 2 X SSC, 1% SDS at 68°C for 30 min. each and twice with 0.1% SSC, 0.5% SDS at 68°C for 30 min. each. The atlas array will be exposed overnight and the results will be compared with the known distribution of genes.

Imaging and quantitation of the cDNA microarray: Imaging data from the Storm PhosphorImager were downloaded into a Microsoft Excel spreadsheet. Duplicate data points were ratio'ed to the ubiquitin standard. Data were then analyzed using the Stanford University ScanAlyze software. These data were also evaluated in parallel with the PSCAN program for point identification and with the JMP program for graphical organization.

Statistical Data mining from cDNA arrays: The first strategy we employed is embodied in the GRASP methodology (Gene Ratio AnalysiS Paradigm, Srivastava et al, 1999). The GRASP algorithm allows us to specify the changes in specific intensities of given genes which are greater or less than one standard deviation (S.D.) from the average

changes of all genes in the entire array. This technique vastly increases the statistical power of the analysis.

4.a.2. Analysis of members of the ANX7 signaling pathway involved in suppression of breast cancer cell growth and apoptosis using protein specific antibodies and Western blot analysis

Rationale: Overexpression of wt-ANX7 causes breast cancer cells to undergo apoptosis. In order to follow the signaling pathway for Anx7 action in breast cancer cells, we investigated the effect of wt-Anx7 on the proteins that are specifically involved in apoptosis using commercially available antibodies against the known proteins. The role of calcium was examined using the dominant negative mutant, ANX7J.

Experiment: Cell extracts were prepared from breast cancer cell lines and cells transfected with 1.0 or 2.0 μg of pAd-CMV (control), ANX7 and ANX7J. Briefly, the cell pellet was resuspended in 200 μl extraction buffer containing 20 mM HEPES (pH

Table 2: Proteins affected by altered expression of *ANX7* or *P53*

	MDAMB-435 Cells					MDAMB-231 Cells				
Protein	*parental*	*control*	*ANX7*	*J*	*P53*	*parental*	*control*	*ANX7*	*J*	*P53*
PARP: Poly(ADP-ribose) polymerase	-	-	-	-	-	-	-	-	-	-
RIP: Receptor Interacting Protein	-	-	-	-	-	-	-	-	-	-
FAS: Factor activating exoenzyme	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BCL-2: B cell leukemia/lymphoma protein2	-	-	-	-	-	-	-	-	-	less
Cas: Cellular Apoptosis Susceptibility gene	-	-	-	-	-	-	-	-	-	less
P53: tumor suppressor gene	-	-	-	less	over	-	-	over	over	over
Casp3: Caspase3	-	-	-	-	-	-	-	less	over	less
GAPDH: glyceraldehyde3-phosphate dehydrogenase	over	over	over	over	over	-	-	-	-	-
Casp2: Caspase 2	-	-	-	-	-	-	-	-	-	less
Casp7: Caspase 7	-	-	-	-	less	-	-	-	-	less
BAX: BCL2 associated X protein	-	-	-	-	-	-	-	-	-	-
TRADD: TNFR-1associated death domain protein	-	-	-	-	-	-	-	-	-	-
BAD: Cell death repressor	-	-	over	-	-	n.d.	n.d.	n.d.	n.d.	n.d.
DFF45: DNA fragmentation factor	-	-	-	-	-	-	-	-	-	less
NIP1: Bd family protein	-	-	-	-	-	-	-	-	-	-
MCL-1: Myeloid cell leukemia protein	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
hILP: Human IAP-like protein	-	-	-	-	-	-	-	over	over	less
FADD: Apoptosis associated protein	-	over	less	less	over	-	over	-	-	less
Cyclin E: Cycline E	-	-	-	-	-	-	-	over	over	less
Fas ligand: Factor activating exoenzyme ligand	-	-	-	-	-	-	over	over	over	less
BCL-X: Apoptosis regulator	less	over	-	-	-	less	less	over	over	less
Apaf1: Apoptotic protease-activating factor1	-	-	-	less	-	-	less	less	less	-
ANX7: Annexin VII	-	-	over	-	-	-	-	over	over	less

n.d.: not detected; - no change in expression

7.9), 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol and protease inhibitor cocktail (Roche, Lewes, UK) and incubated on ice for 30 min with intermittent mixing. The extract was centrifuged for 20 min at 4°C at 10,620*g*. The supernatant was directly used for Western blot analyses. Proteins were separated on a 10% SDS-polyacrylamide gel and transferred electrophoretically to a Sequi-Blot PVDF membrane (Bio-Rad) in 20 mM sodium phosphate buffer (pH 6.8). The filter was blocked in 5% BSA in TST, washed in TST buffer alone and incubated at room temperature for 1.5 hr with the respective antibody specific for the proteins that are shown in **Table 2** at the recommended dilution. The filter was washed in TST and incubated with a 1:10,000 dilution of second antibody HRP conjugate. After extensive washing in TST, the blot was incubated with SuperSignal West Pico Chemiluminescence kit (Pierce, Rockford, IL) reagents and exposed to XAR .lm.

The results show that altered ANX7 has differential effects on p53 protein, caspase-3, BAD, FADD an apoptosis associated protein, cyclin E, Fas ligand, BCLX, APAF-1 in the metatsatic and non-metastatic breast cancer cells

Interpretation: We have identified several proteins that are involved involved with apoptosis differentially expressed in the wt-ANX7 transfected tumor cells compared to the dominant negative mt-ANX7J using western blotting.

We are in the process of validating the results using breast cancer cells transfected with vector alone, wt-ANX7, ANX7J or p53 at different time points.

KEY RESEARCH ACCOMPLISHMENTS

- Generation of multi-ANX7 expression vectors for evaluation of biochemical functions of ANX7.
- Identification of the dominant negative mutant against calcium and GTP in the anx7 coding domain which are necessary for biochemical functions of ANX7.
- Generation of recombinant adenoviral vectors containing the dominant negative mutants
- Evaluation of altered ANX7 expression in breast cancer cell growth show that calcium associated function with this identified mutation is mechanistically involved in the tumor suppression phenotype and down-regulation of ANX7 could prove to be therapeutic
- Evaluation of altered ANX7 expression in breast cancer cell growth shows further that ANX7 controls all three subtypes of IP3 Receptor expression
- Identification of the downstream targets and signaling pathway of ANX7 in apoptosis and suppression of breast cancer cell growth using cDNA microarray
- Identification of members of the signaling pathway of ANX7 in apoptosis and suppression of breast cancer cell growth using protein-specific antibodies and Western blot analysis

REPORTABLE OUTCOME

1. We showed that regulating ANX7 levels could be therapeutic. In addition, we identified the downstream targets and signaling pathway of ANX7 in apoptosis and suppression of breast cancer cell growth using cDNA microarray.
    - A manuscript is under preparation.
    - The results were presented in USUHS research day, 2003 as part of the plenary session talk and in the poster
    - The results were also presented in the "3rd Annexin conference held in Canada, 2003 in the plenary session
    - The results formed the preliminary data for the grant, "ANX7 as a molecular target for breast cancer" that I submitted to NIH on October 1st

CONCLUSIONS

We have generated and identified the dominant negative mutants against calcium and GTP in the anx7 coding domain which are necessary for biochemical functions of ANX7. We have generated the effective adenoviral constructs containing the dominant negative mutants and wt-ANX7 and have shown that altered ANX7 expression during breast cancer cell growth involves calcium and down-regulation of ANX7 could prove to be therapeutic. Additionally, we confirmed the role of calcium by determining ANX7's control on all three subtypes of IP3 Receptor expression. Using cDNA microarray and Western blot analysis, we have identified the downstream targets and signaling pathway of ANX7 in apoptosis and suppression of breast cancer cell growth. Taken together, these data indicate that ANX7 suppresses breast cancer cell growth and calcium plays a role via IP3-Receptor. In addition, we have identified the members of the ANX7 signaling pathway. Comprehensive effort is underway to validate the results that will further elucidate the ANX7 dependent calcium signaling pathway in breast cancer cells.

REFERENCES

Caohuy, H., Srivastava, M., and Pollard, H.B. (1996) GTP-activation of membrane fusion protein anx7 (Annexin VII) and detection of Ca^{2+} -activated GTPase activity in vitro and in vivo. Proc.Nat.Acad.Sci.(USA) 93:10797-10802.

Creutz, C.E., Pazoles, C.J., and Pollard, H.B. (1978) Identification and Purification of an Adrenal Medullary Protein (Synexin) That Causes Calcium Dependent Aggregation of Isolated Chromaffin Granules. J. Biol. Chem., 253:2858-2866.

Creutz, E.C., Pazoles, C.J., and Pollard, H.B.(1979) Self-Association of Synexin in the Presence of Calcium: Correlation with Synexin-Induced Membrane Fusion and Examination of the Structure of Synexin Aggregates. J. Biol. Chem., 254:553-558.

Raynal, P. and Pollard, H.B. (1994) Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium-and phospholipid-binding proteins. BBA Biomembranes 1197: 63-93.

Srivastava, M. Eidelman, O. and Pollard, H.B. (2000). Gene expression microarray pharmacogenomics of wt and mutant CFTR, and of the CF drug CPX. Mol. Medicine 5, 753-767.

Srivastava, M., Bubendorf, L., L., Nolan, L., Glasman, M., Leighton, X., Koivisto, P., Willi, N., Gasser, T., Kononen, J., Sauter, G., Kallioniemi, O.P., Srivastava, S. and Pollard, H.B. (2001) ANX7, a candidate tumor-suppressor gene for prostate cancer. Proc. Natl. Acad. Sci. 98, 4575-4580

Srivastava, M., Bubendorf, L., L., Nolan, L., Glasman, M., Leighton, X., Miller, G., Fehrle., W., Raffeld, M., Eidelman, O., Kallioniemi, O.P., Srivastava, S. and Pollard, H.B. (2001) ANX7 as a biomarker in prostate and breast cancer progression. Disease Markers 17, 115-20

Srivastava, M., Kumar, P., Leighton, X., Glassman, M., Goping, Eidelman, O. and Pollard, H.B. Influence of the Anx7 (+/-) Knockout Mutation and Fasting Stress on the Genomics of the Mouse Adrenal gland. Ann.N.Y.Acad. Sci., 2002; 971:53-60

Srivastava, M., Eidelman, O. Leighton, X., Glassman, M., Goping, and Pollard, H.B. Anx7 is Required for Nutritional Control of Gene Expression in Mouse Pancreatic Islets of Langerhans. Mol. Medicine. 2002; 8, 781-797.

Srivastava, M., Glasman, M., Leighton, X, Miller, G., Montagna, C., Reid, T., and Pollard, H.B. Genomic instability and gene expression profile in cancer prone Anx7(+/-) knockout mouse and cell line models. Proc Natl Acad Sci U S A. 2003; (In Press).